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Stem Cells in Hepatocarcinogenesis: Evidence from Genomic Data

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Abstract

Increasing evidence suggests that many, perhaps all solid tumors contain a subset of cells that possess functional properties similar to the normal tissue stem cells, including self-renewal, unlimited proliferative capacity, and pluripotency. The hierarchical cancer model that places a cancer stem cell (CSC) population at the apex of tumor formation is based on this notion. The cancer stem cell hypothesis posits that CSCs are responsible not only for tumor initiation, but also generation of metastasis and local recurrence after therapy. Current definitions of the CSC are based only on functional properties regardless of potential cellular origin. Histopathology investigations of chronic liver diseases and experimental studies support the existence of CSCs in liver cancer. In particular, recent advances in microarray technologies utilizing integrative comparative genomic analysis of human hepatocellular carcinoma specimens, cancer cell lines, and transgenic models establish the molecular similarities between CSC and normal tissue stem cells and highlight the importance of CSC for the prognosis of liver cancer patients. The results have also uncovered the key “stemness” and oncogenic pathways frequently disrupted during hepatocarcinogenesis providing the basis for identifying novel therapeutic targets against CSC.

Keywords

Hepatocellular carcinoma; stem/ progenitor cells; oval cells; comparative functional genomics; cancer stem cells; hepatocarcinogenesis

The concept that many solid tumors adopt a hierarchical organization is being increasingly recognized. Although the idea that only a small subpopulation of cells within each tumor possesses the capacity to generate tumors in vitro and in vivo is not new, research on the “cancer stem cells” (CSCs) in solid tumors mushroomed only in the last decade.^{1–7} In contrast to the traditional stochastic cancer model, the hierarchical model postulates that cancer is a genetically generated disease that is driven by epigenetic changes originated in a minor cell population (<1%) possessing stemness features (commonly referred to as CSC) and occupying the apex of the tumor hierarchy. Similar to the phenotypic diversity of normal adult tissues maintained by the tissue stem cells, the CSC model is helpful to explain the heterogeneity observed within many clonally derived tumors including liver cancer.^{7–10} Although the CSC may have functional properties similar to the normal tissue stem cells,

including self-renewal and differentiation capacity, the cancer stem cell hypothesis does not make any assumptions about a putative origin of these cells.¹¹

Although numerous hypotheses have attempted to explain how cancer stem cells and tumor heterogeneity originate,^{7,12} the general concepts of carcinogenesis consider at least three different scenarios: (1) differentiation arrest of adult tissue stem cells and/or progenitor cells, (2) dedifferentiation of mature cells, and (3) transdifferentiation of stem cells from different tissues, for example, bone marrow. A combination of all three scenarios may provide another conceivable explanation. The relative contribution of each postulate may depend on number of factors, including type of cancer, tissue microenvironment, and the contributing mutagen(s).

It is axiomatic that comprehensive definition and characterization of the putative cancer stem cells and their cellular origin will improve both the understanding of tumor biology and the prospects of developing new cancer therapies. Because classical therapeutic regimens target proliferating cells, they are unlikely to target the CSC, which are thought to be low turnover/quiescent cells.^{13–15} A detailed characterization of CSC would include the clarification of the underlying molecular, genetic, and epigenetic mechanisms responsible for tumor initiation, seeding of metastasis, and local recurrence that are currently attributed to the CSC.^{12,16} Transcriptome analyses utilizing microarray technologies have significantly advanced the field of cancer research in the last decade. Progressive application of these technologies for investigating CSCs seems particularly relevant to address several of the unresolved issues regarding the role of CSC in liver cancer.^{17,18}

CELLULAR ORGANIZATION OF LIVER AND HEPATOCARCINOGENESIS

The human liver is anatomically divided into different macroscopic and microscopic units: lobes and hepatic lobules. The functional unit or hepatic lobule is limited by six portal triads containing the hepatic artery, portal vein, and bile duct and centered on central vein. Venous blood from the intestine enters the liver from the portal vein and interconnects the portal triads with the systemic circulation while passing through fenestrated endothelial sinusoids that are tightly surrounded by the basolateral side of hepatocytes along with the resident nonparenchymal cells of the liver (e.g., stellate cells, Kupffer cells, immune cells, etc.). The apical sides of the hepatocytes form the bile canaliculi from which bile drains back toward the bile ducts. The most terminal branches of the biliary epithelium, denoted as Canals of Hering, are believed to be the niche for hepatic stem/progenitor cells (a more detailed description of the liver anatomy is provided elsewhere).^{19,20} Three types of liver cells, hepatocytes, cholangiocytes, and adult stem/progenitor cells, are commonly regarded as the primary targets of malignant transformation in the liver.

The extensive regenerative and proliferative capacity of mature hepatocytes is well recognized. Elegant experimentation with serial transplantations has shown that hepatocytes can undergo at least 69 doublings without losing functional potential; additionally, hepatocytes have a long lifespan.^{21–23} Because these characteristics indicate that mature hepatocyte possesses intrinsic stem cell-like traits, it could be a target of malignant transformation.

The notion that hepatic stem/progenitor cells can drive hepatocarcinogenesis and be a source of CSC is historically well documented. Experimental induction of liver stem/progenitor cells in rodents has been extensively studied in liver injury models as well as in liver carcinogenesis. In agreement with the cancer stem cell model, the application of a variety of experimental protocols in rodents resulted in activation and proliferation of adult liver stem cells (often referred to as oval cells) and almost as frequently in carcinogenesis. Oval cells are putative liver stem/progenitor cells and were first described by Opie in 1944, and later

by Farber.^{24,25} Oval cells are thought to reside in the Canals of Hering and, at a minimum, give rise to both cholangiocytes and hepatocytes.^{26–28} Activation and proliferation of hepatic progenitor cells have been reported in many precancerous conditions, such as chronic inflammation (hepatitis B and C, alcoholic hepatitis, and steatohepatitis).^{29–31} Therefore, liver stem/progenitor cells could be another source of CSC in HCC.

Although convincing evidence supporting a role for bone marrow-derived stem cells in the process of malignant transformation in liver is yet to be provided, there are data to suggest that bone marrow-derived cells may take part in liver regeneration and therefore should also be taken into consideration.^{32–35} For example, transplantation of adult bone marrow cells has been reported to restore liver tissue and function in fumarylacetoacetate hydrolase (FAH-) deficient animals,³⁶ although more recent data has demonstrated that the contribution of bone marrow cells to liver recovery was due to the fusion with the host hepatocytes.³⁷ Together, these observations emphasize that many different cell types may be the potential targets of a transforming event(s), thereby contributing to hepatocarcinogenesis (Fig. 1). The different cellular origin may provide a potential explanation for the heterogeneity of CSCs and the phenotypic diversity of liver cancer.

EVIDENCE FOR A STEM CELLS ORIGIN IN LIVER CANCER

In the past, a plethora of transgenic mouse models and a variety of different chemical- or oncogene-induced carcinogenesis models were used to directly determine the role of stem cells in hepatocarcinogenesis. The common conclusion of the early studies was that HCC can originate from adult liver stem/progenitor cells.^{38–42} Also, there is evidence for the stem/progenitor cell origin in human HCC. Thus, extensive stem/progenitor cell proliferation frequently referred to as “ductular” reaction can be observed in preneoplastic conditions of the liver, such as chronic inflammation. Similar to oval cell activation in rodents, the extent of ductular reaction correlated with the severity of the underlying liver disease.^{43–45} Furthermore, after malignant conversion a substantial number of HCCs demonstrate “bipotential” characteristics: tumor cells coexpressing biliary and hepatocytic markers, such as cytokeratin 7 (CK7), CK19, OV6, α -fetoprotein (AFP), and albumin.^{23,46} The presence of these markers was associated with a more aggressive phenotype and bad clinical outcome.⁴⁷ An integrative study from our laboratory performed on a rat model of HCC further validated CK19 as a prognostic marker of early preneoplastic lesions suggesting a progenitor origin of these tumors. In addition, genomic analysis showed that CK19-associated gene signature stratified HCC patients according to clinical outcome.⁴⁸

Other recent studies suggest that disruption of interleukin-6 (IL-6) and transforming growth factorbeta (TGF- β) signaling pathways in adult liver tissue stem cells was a key event leading to the transformation of pluripotent liver stem cells.⁴⁹ Another study reported that c-KIT (encodes a proto-oncogenic receptor tyrosine kinase) expressing proliferating liver progenitor cells were the targets of transforming events in a setting of chronic liver injury.⁵⁰ However, convincing evidence for the clonal origin of liver cancer from the stem cell compartment is still missing.

GENOMICS IN HEPATOCARCINOGENESIS

Over the last decade, the rapid advances in microarray technology have opened a new genomics era in biology and cancer research in particular. Whole genome transcriptomics and other array-based analyses provided more global and unbiased tools to study mechanisms of carcinogenesis in different tumor types.⁵¹ In the liver, microarray analysis uncovered a variety of molecular signatures, signaling pathways, and gene sets associated with human liver cancer.^{52–55} Significantly, in addition to antiapoptotic and proproliferative signaling, many of the disrupted pathways in HCC have been shown to be involved in stem

cell maintenance and self-renewal, for example, Wnt/ β -catenin, TGF- β , Met (hepatocyte growth factor receptor), Hedgehog, as well as to be activated in adult liver progenitor cells upon regenerative stimuli.^{21,56–58} Because most of the HCCs develop in the background of chronic inflammatory driven regenerative cycles, activation of these pathways may be indicative of a stem cell origin at least in some liver tumors.⁵⁹ Clear prognostic significance of their disruption in different subtypes of liver cancer further confirms this finding.⁶⁰ Recent investigations used a more global approach and systematically combined transcriptional programs from different studies to construct a compendium of genes important for embryonic stem cells (ESCs) and adult tissue stem cells.^{61,62} The authors found activation of ESC-like transcriptional programs in aggressive human epithelial cancers including liver cancer. Interestingly, activation of the MYC oncogene was particularly important both for the tumor initiation and the reactivation of the ESC-like module in normal and cancer cells. This observation confirms the results from murine and human studies indicating the critical role for MYC in hepatocarcinogenesis.^{63,64} More recent study using transcriptomic analyses of cirrhotic and dysplastic nodules and early HCC further demonstrated activation of the MYC-associated gene sets in early HCC consistent with the central role of MYC in driving malignant conversion of preneoplastic liver lesions.⁶⁵ The subsequent classification based on the MYC target genes expression clearly discriminated between preneoplastic stages and early HCC.

Comparative genomic investigations of human HCC provided evidence supporting stem/progenitor cell origin in a subset of human liver cancer.^{66,67} In this study, gene expression data from rat fetal hepatoblasts (HB) and adult hepatocytes were integrated with the gene expression data from human HCC. HCCs, which shared gene expression pattern with HB (fetal progenitor cells), were profoundly different from other prognostic subtypes of HCC (Fig. 2).⁵³ The gene expression signature of the HB subtype of HCC included hepatic oval cell markers and was strongly associated with worse prognosis, consistent with hepatic progenitor cell origin.⁶⁷ The prognostic implication of a progenitor signature was independently confirmed by others.⁵⁴ Similarly, classification of HCC based on the gene expression of the onco-fetal molecules, epithelial cell adhesion molecule (EpCAM) and AFP revealed distinct HCC subtypes resembling consecutive stages of hepatocyte differentiation. Gene expression signature of EpCAM⁺/AFP⁺ HCC was associated with poor survival and was characterized by activation of WNT- β -catenin, TGF- β , epidermal growth factor (EGF), and p53.

Taken together, the transcriptomic analyses of HCC have significantly contributed to the understanding of the cellular origin of liver cancer and identified signaling pathways likely associated with progenitor cells. Integrative approaches further showed a clear prognostic significance of the progenitor cell-like signatures making them valuable tools for generating novel preventive, diagnostic, and/or therapeutic strategies for HCC patients.

PROSPECTIVE ISOLATION AND DEFINITION OF HEPATIC CANCER STEM CELLS

In the last years, considerable progress has been made in the prospective isolation and characterization of potential cancer stem cells in human HCC. Cancer stem cells are defined by at least three distinct properties: (1) selfrenewing capacity—the ability to undergo symmetric and asymmetric division, thereby “infinitely” repopulating the cancer stem cell pool; (2) differentiation capacity—the ability to recapitulate all cell types of original tumor; and (3) tumor-initiating capacity—the ability to propagate tumors when transplanted into a proper environment.¹¹ Based on these definitions, two general approaches have been established for the isolation of CSC. First, and the most commonly employed, is an immunogenic approach based on the prospective isolation of a CSC using cell surface

antigens, which are frequently adapted from hematopoietic stem cells. The second approach utilizes characteristics that CSCs share with normal stem cells, such as sphere forming ability, chemoresistance, self-renewal, asymmetric division, and “stemness” gene signatures. Both approaches have been successfully used to isolate CSCs in liver cancer (Table 1). All isolated cells showed essential cancer stem cell characteristics, such as self-renewal, multipotency, and extensive proliferation capacity, regardless of using functional and/or antigenic-based isolation procedures. Consistent with the CSC hypothesis and in agreement with observations from other types of tumor, the liver CSCs comprise only a minor highly aggressive subpopulation within an original tumor.^{68–72}

Currently, there exist limited genomic data on prospectively isolated putative liver CSCs. The transmembrane protein Prominin1 (CD133) appears to be useful for isolation of liver CSCs.⁷⁰ Available gene expression data of CD133⁺ cells show activation of stemness genes involved in Wnt/ β -catenin, NOTCH, and Hedgehog signaling pathways.⁷³ Another frequently used CSC marker for isolating liver CSCs is EpCAM, also known as ESA and TACSTD1.^{71,74,75} As mentioned above, classification of HCC patients based on the EpCAM expression has a prognostic significance. HCC classified as a hepatic progenitor cell subtype (HpSC) showed activation of the stem cell and self-renewal pathways, including TGF- β , Wnt/ β -catenin, PI3K/Akt, and expressed stem/progenitor cell and CSC markers, such as *KRT19* (CK19), *TACSTD1* (EpCAM), *AFP*, *DKK1*, *DLK1*, and *PROM1* (CD133). Conversely, the mature hepatocyte subtype (MH) displayed the activation of the gene sets associated with the hepatocyte functions, such as metabolism, complement system, and coagulation.^{54,71} Furthermore, the prospectively isolated EpCAM⁺ cells exhibited an adult stem cell-like gene expression profile as demonstrated by cluster analysis based on the TaqMan human stem cell pluripotency array, whereas EpCAM[−] cells were closer to MH subtype HCC.

Another technique to isolate cells with CSC properties is based on the capacity to exclude the vital dye Hoechst 33342, which defines the so-called side population (SP).⁷⁶ Gene expression analysis by reverse transcription polymerase chain reaction (RT-PCR) performed on the SP cells showed that the genes important for stemness, differentiation, as well as chemoresistance were differentially expressed as compared with the corresponding non-SP.^{68,69} Subsequent work has demonstrated that BMI1, a part of the Polycomb group complex PRC1 (protein regulator of cytokinesis) known for its contribution to stem cell self-renewal, has a critical role in maintaining and propagating SP population in liver cancer, suggesting the potential stem cell origin of CSC.^{77–79} Gene expression profiling of SP cells from two different HCC cell lines further showed activation of oncogenic and stemness pathways, such as Wnt and Jun signaling.

Our transcriptomic analysis of epigenetically modified SP cells using the DNMT1-inhibitor Zebularine provides further evidence for a potential stem cell origin of liver CSC (our unpublished data).⁸⁰ Signaling pathways important for developmental processes, such as Wnt/ β -catenin and IL6, and stem/progenitor cell markers, including CK19, SOX9, SOX4, DMBT1, MED12, and AMD1, were differentially expressed in the isolated SP cells. Additionally, gene set enrichment analysis (GSEA) revealed strong enrichment of gene sets important both for the tissue stem cells and embryonic stem cells. Consistent with the findings from the module map, integrative analysis of the generated CSC signature showed a clear association with poorly differentiated tumors and a bad prognosis of HCC patients.

Although the available data on genomics of prospectively isolated liver CSCs are still limited and overlap between gene expression data and antigenic characteristics from different studies is low, the findings discussed above emphasize the common patterns supporting the existence and importance of CSCs in human HCC. Furthermore, the

prognostic impact of the CSC gene expression signatures may suggest a paradigm shift from the traditional therapies to those aimed at eradicating CSCs.⁸¹ Taken together, the existing evidence from microarray-generated molecular classifications of HCC suggests a potential stem cell origin in a subpopulation of human HCC and underlines prognostic implications of the prospective analysis of putative cancer stem cells. Because the heterogeneity of human liver cancer disease may be related to CSC origin, the hepatic CSC cannot be defined by the expression of a single marker. A combination of different functional and antigenic approaches should be used for CSC identification and characterization (Fig. 3).

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ABBREVIATIONS

AFP	α -fetoprotein
CK	cytokeratin
C-Kit	encodes a proto-oncogenic receptor tyrosine kinase
CSC	cancer stem cell
ESC	embryonic stem cell
EGF	epidermal growth factor
EpCAM	epithelial cell adhesion molecule
FAH	fumarylacetoacetate hydrolase
GSEA	gene set enrichment analysis
HB	hepatoblast
HCC	hepatocellular carcinoma
HpSC	hepatic progenitor cell
IL	interleukin
MH	mature hepatocyte
MET	MET proto-oncogene (hepatocyte growth factor receptor)
PRC	protein regulator of cytokinesis
RT-PCR	Reverse transcription polymerase chain reaction
SP	side population
TGF	transforming growth factor

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Figure 1.

Potential cell types of origin of liver cancer stem cells (CSCs). The scheme illustrates the cellular organization of a portal triad consisting of a portal vein (PV), hepatic artery (A), and bile duct (BD). The most terminal branches of the biliary tree are referred to as Canals of Hering. CSCs can originate from the stem/progenitor cells thought to reside in Canals of Hering. They extensively proliferate upon adequate stimuli commonly present in hepatocarcinogenesis, for example, liver damage. CSCs can also originate from mature hepatocytes known to possess stem-like characteristics, such as proliferation capacity and longevity. Hepatocytes are continuously exposed to exogenous and endogenous stress and could be targets for transforming events. Lastly, bone marrow-derived stem cells could be a source of hepatic CSC through transdifferentiation.

Figure 2.

Genomic evidence for a progenitor cell origin in liver cancer. The comparative functional genomic analysis of 139 human hepatocellular carcinomas (HCCs) and rat hepatoblasts/hepatocytes identified 22 human HCCs that showed similar expression pattern to stem-like hepatoblasts (HBs; designated as HB type) (adapted from Lee et al⁶⁷). Kaplan-Meier plots showed that patients with HB subtype HCC had worse survival and a higher frequency of recurrence. The study highlights the potential stem cell origin in a subtype of HCC and demonstrates clinical relevance of this finding.

Figure 3.

Integrative genomic analysis and its implication for cancer cell research (CSC). Genomic and epigenomic analysis of putative CSC can be used for cross comparison and validation using independent data from different sources (cell culture, human patients, and mouse models). The data can then be integrated and subjected to different analysis to test whether the isolated cells fulfill CSC criteria, i.e., stemness activation of relevant signaling pathways and gene modules. After validation, CSC can be then used for detailed characterization of the key molecular changes, identification of the cellular origin, and stratification of cancer patients. The obtained data can be used to identify new therapeutic targets to specifically eliminate CSC. HCC, hepatocellular cell carcinoma.

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Commonly Used Markers to Identify Liver Cancer Stem Cells (CSCs)

Table 1

Marker/Method	Frequency	Minimal Cell Number Required for Tumor Initiation	Other Solid Tumors	Reference (for Liver CSC)
SP	0–28%	1000	Lung, breast, pancreas	69,78,82
CD133	0–65%	1000	Breast, colon, pancreas, endometrium, prostate, brain	70
CD133/CD44	0.1–1.9	100	Colon, breast	83
CD133/ALDH1	0–56%	500	Colon, breast	73
CD90	0–2.5%	500	Prostate stroma	72,84
EpCAM	0–99%	200	Breast, pancreas, colon	71

SP, side population; EpCAM, epithelial cell adhesion molecule.